Reference: *Biol. Bull.* **188:** 267–280. (June, 1995)

Cytological Analysis of the Urn Cell Complex of *Sipunculus nudus* Before and After Serum-Induced Secretion

SANTO V. NICOSIA[1] AND JANICE M. SOWINSKI[2]

[1]*University of South Florida College of Medicine, Department of Pathology and H. Lee Moffitt Cancer Center, Tampa, Florida 33612; and* [2]*Department of Pathology and Toxicology, SmithKline and French Laboratories, Philadelphia, Pennsylvania 19101*

Abstract. This study analyzes the cytology of the urn cell complex (UCC) of *Sipunculus nudus*, an invertebrate cell model for humorally regulated mucus secretion. An unstimulated UCC is composed of a vesicle cell and a ciliated cell joined together by desmosomes. Another cell population (third-type cells) is frequently associated with ciliated cells. Vesicle cells are thin, have few mitochondria or lipid droplets, and enclose a bubble-like cavity containing microfibrillar material. Ciliated cells contain several rows of cilia that are anchored by prominent rootlets and propel UCCs forward. Five to six concentric bundles of microfilaments are distributed along the outer convexity of ciliated cells and may have a role in the plasticity of the UCC. Many fibrillar deposits that lack a demonstrable limiting membrane are distributed around intracytoplasmic vacuoles facing the mouth-like opening of the UCC. These deposits are reactive to periodic acid-Schiff and resistant to diastase. After stimulation with serum, they appear to migrate through the ciliated cell's plasma membrane, contributing to the formation of a secretory tail. Discharge of secretory material is not observed in third-type cells, which instead contain lysosome-like granules and autophagic-like vacuoles and become displaced distalward by the emerging tail of the UCC. This study indicates that formation and elongation of the UCC secretory tail are functions of ciliated cells.

Introduction

Mucous secretions regulate bacteria and sperm migration and protect against foreign irritants. The control of mucus secretion in non-innervated cells, such as those lining the respiratory and reproductive tracts, is poorly understood (Chantler and Debruyne, 1977; Richardson and Phipps, 1978; Nicosia, 1986). Using an invertebrate mucociliary cell model, Bang and Bang (1962, 1979) reported that one or more mucus-releasing macromolecule is present in body fluids under physiologic and pathologic conditions. A similar factor or factors may also be present in vertebrate external secretions and sera (Bang and Bang, 1974, 1979; Mastroianni *et al.*, 1979; Nicosia, 1979; Kurlandsky *et al.*, 1980; Nicosia *et al.*, 1982). This possibility has indeed been verified with cat tracheal cells (Hall *et al.*, 1978) and rabbit endocervical mucous cells (Nicosia *et al.*, 1982; Nicosia, 1986).

The cell model used by Bang and Bang (1962, 1972) is derived from the benthic coelomate *Sipunculus nudus* (Linnaeus). This marine invertebrate belongs to a phylum of sipunculids (peanut worms) with an unsegmented, cylindrical body and a retractile anterior introvert (Hyman, 1959). A characteristic of this sipunculid is the presence of a large number of circulating mucociliary cells, or urn cell complexes (UCCs), within its coelomic cavity (Catacuzéne, 1928). Swimming freely within such a cavity, UCCs play an important role in the sipunculid immune defense by releasing sticky, mucoid tails that promptly trap invading bacteria, thereby curtailing infection (Bang and Bang, 1974, 1975).

Morphological data on the UCCs of *S. nudus* derive mostly from the elegant light microscopic observations of Bang and Bang (1962, 1964, 1976, 1980). These studies indicate that the UCC is composed of two cells, a vesicle cell and a saucer-shaped ciliated cell, fitted together like an acorn into its cap. A third type of cell is often contig-

Received 10 May 1994; accepted 24 March 1995.

uous with the former two cells and may contribute to or regulate mucus secretion. Only two brief reports on the ultrastructure of the UCC of *S. nudus* have been published (Nicosia and Sowinski, 1979; Reissig *et al.*, 1979). A more detailed study of UCCs and of other coelomic cells has been carried out in *Phascolosoma agassizii*, but significant differences appear to exist between the cell complexes of that worm and those of *S. nudus* (Dybas, 1976, 1981a).

The present study was carried out to verify the multicellular nature and cell relationships of UCCs in *S. nudus* and to analyze the cytology of these cells before and after serum-induced mucus release.

Materials and Methods

Animals

Adult specimens of *Sipunculus nudus* were obtained from two sources. Some worms were dug from the sand at low tide in their natural habitat in Salerno, Italy. Worms that were traumatized were discarded. Several dozen worms were placed in oxygenated, airtight containers partially filled with seawater and fresh sand and flown to our laboratory. Healthy worms were immediately transferred to an aerated aquarium (Instant Ocean Inc., Wicklife, OH) with a good cover of sand and recirculating, filtered seawater (FSW). Other worms were originally collected at Loquemeau (Finistére, France) and then maintained at the Marine Biology Laboratory, Woods Hole, Massachusetts, in large tanks with circulating seawater.

Harvesting of urn cell complexes

Suspensions of UCCs were obtained by withdrawing 2–3 ml of coelomic fluid with a 25-g needle through an opening at the posterior end of each female worm. Fluid aliquots (1.0 ml) were stored in capped, 6.0-ml plastic tubes (Falcon-Bioquest, Cockersville, MD) held in an upright position at 4°C.

In vitro *bioassay for serum-induced secretion*

Five microliter samples of coelomic fluid supernatant, each containing 50–100 UCCs, were incubated in a Clay-Adams microculture slide dish (Thomas Co., Philadelphia, PA) with 20 μl of FSW, pH 7.9, or with 20 μl of rabbit serum previously diluted 1:10 with FSW. Following the protocol outlined by Bang and Bang (1979), diluted serum was heated at 85°C for 5 min, then stored at 4°C in a sterile container. Seawater was boiled for 5 min, filtered through a 0.22-μm Millipore filter (Millipore Co., Bedford, ME) and also stored at 4°C. UCCs in FSW alone or with 1:10 treated serum were incubated from 0–30 min at room temperature inside a humidified dish. After 0, 2, 5, 10, 20, and 30 min, each UCC preparation was observed under a Wild M20 phase-contrast microscope. Secretory tails released by UCCs were measured with a calibrated crossline ocular disk at a final magnification of 125× (Fig. 1A). Ten incubations were carried out for each investigated time period, with an average of 50 UCCs evaluated to calculate secretory tail length. The secretory response of each cell preparation was expressed as mean tail length in micrometers.

Cytochemical studies

After each incubation, UCCs were resuspended in FSW and pelleted (1400 rpm for 10 min) onto a microscope slide in a Shandon cytocentrifuge (Shandon Southern Instruments, Sewickley, PA). After air-drying, cells were processed for cytochemical evaluation of metachromatic material, lipid, glycogen, glycoproteins, and sugars. For observations of general morphology (with a light microscope) and for studies of metachromasia, cells were fixed in 10% formalin in FSW and stained with 1:5000 methylene blue. For demonstration of lipids, cells were fixed in Baker's fixative and stained with Sudan black B (Lillie, 1965). As controls, some cells were stained after incubation in ether: ethanol (1:1) to extract lipids. For demonstration of glycogen and glycoproteins, cells were fixed in formalin–acetic-acid–alcohol and stained with periodic acid-Schiff (PAS) reagents (Luna, 1968) before and 30 min after digestion with diastase (0.1% in 0.16 *M* phosphate buffer, pH 6.0) at room temperature to remove glycogen. For demonstration of sugar residues in secretory tails of stimulated UCCs, these cells were fixed in 10% formalin for 5 min, rinsed three times in FSW, and incubated for 10 min with fluorescein isothiocyanate-conjugated (FITC) *Ricinus communis* agglutinin (RCA_1) or *Lotus tetragonolobulus* agglutinin (LTA). Both lectins were purchased from Sigma (St. Louis, MO) and used at a concentration of 200 μg/ml in FSW adjusted to pH 7.9. Controls for specific lectin binding contained 10^{-1} *M* L-fucose or D-galactose in staining and washing solutions. After washing, cells were covered with a coverslip, using nonfluorescent mounting medium, and viewed under a Leitz Ortholux microscope using excitation and barrier filters for fluorescein fluorescence. Photomicrographs were taken using Kodak Tri-X film.

Electron microscopy

For transmission electron microscopy, UCCs before and after 2, 10, and 20 min of exposure to serum were fixed for 60 min at 4°C in 3% glutaraldehyde buffered with 0.1 *M* sodium cacodylate in FSW. Cells were postfixed with 1% osmium tetroxide, dehydrated in graded ethanols, and embedded in Epon 812 (Luft, 1961). Specimens were step-sectioned with a diamond knife, and 600–800 Å thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed in a Hitachi 12A

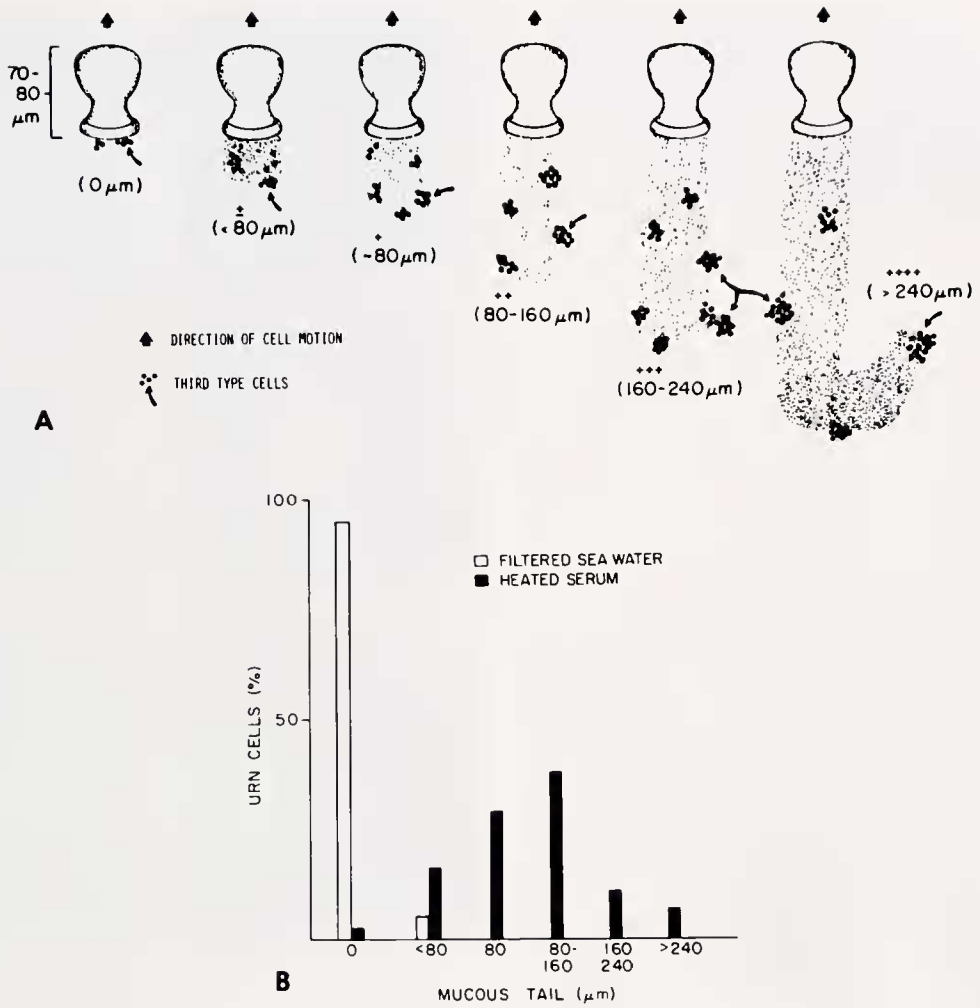

Figure 1. Secretory response of the urn cell complex (UCC) to serum. (A) Schematic diagram. Tails released by UCCs after exposure to heated serum have various lengths (from 0 to > 240 μm) and are easily quantifiable with the aid of an ocular micrometer under phase-contrast microscopy. Large arrows indicate direction of UCC swimming motion. Smaller arrows point to third-type cells, which are progressively displaced distalward by the emerging tails. (B) Typical distribution of secretory responses (expressed as percentage of UCCs with various tail lengths) 20 min after exposure to heated serum (full bars) or filtered seawater (empty bars).

electron microscope at an initial accelerating voltage of 75 kV.

For scanning electron microscopy, UCCs were pipetted onto round coverslips (20 mm diameter, Carolina Biological Supplies, Burlington, NC) previously coated with 0.1% poly-D-lysine (Sigma Chemical Co., St. Louis, MO). Cells were then fixed *in situ* for 60 min at 4°C with a 3% glutaraldehyde buffered with 0.1 *M* sodium cacodylate buffer in FSW. After they were rinsed in the same buffer, cells were dehydrated in graded acetones and critical-point dried in a Tousimis device (Tousimis Inc., Rockville, MD) using liquid Freon as transitional fluid. Coverslips were then mounted onto stubs with a thin layer of conductive silver point (Fullam, Schenectady, NY) and sputter-coated with a layer (~15 nm) of gold-palladium in a Balzer device (Balzer Union, Nashua, NH). Specimens were viewed in a Jeolco SM-35 scanning electron microscope operated at 10 kV with a 0–60°C stage tilt. Photographs were taken using Polaroid 55 P/N film.

Results

Effect of serum

UCCs exposed to 1:10 heated serum extruded distinct secretory tails as early as 2 min after stimulation (Fig. 1A). The length of these tails increased after the ensuing 10 min, reaching a peak after 20 min. The average tail length during the observed intervals was 10 μm at 2 min,

30 μm at 5 min, 55 μm at 10 min, 100 μm at 20 min, and 110 μm at 30 min. After 20 min, a wide distribution of secretory response was observed, with more than 50% of UCCs exhibiting tails 80 μm or longer (Fig. 1B). About 5% of UCCs exposed to serum showed no visible tails. Tails were absent or shorter than 80 μm in UCCs incubated with FSW alone (Fig. 1B).

Light microscopy

Living or fixed UCCs measured from 70 to 80 μm in their longest dimension. The apical, or vesicle, cell contained a single nucleus and was translucent (Fig. 2A). The basal, or ciliated, cell was saucer-shaped, also contained a single nucleus, and was recognized by the active and rapid beating of cilia (Fig. 2A). The site where the neck of the vesicle cell fitted into the ciliated cell was marked in its outer profile by five to six parallel arrays of refractile and wavy "lines" (Fig. 2B). Below this area, the outer aspect of the ciliated cell contained several rows of circumferentially arranged cilia, 5–10 μm long. In oblique and "tail-on" views, the cytoplasmic insertion shafts of these cilia were observed as refractile "ribs" (Fig. 2B–C). In live UCCs, cilia swept very rapidly to ensure forward motility and also to sweep surrounding particles. The concave or inner aspect of the ciliated cell was devoid of cilia, smooth, and formed a mouth-like opening at the junction with the lower region of the vesicle cell (Fig. 2D). A cluster of round cells was frequently found at or near the lower aspect of UCC. These third-type cells measured 7–9 μm in diameter and were best observed in lateral views of UCCs (Fig. 2B and E). These views revealed 5–10 or more cells often surrounded by the scanty secretory material present on the underside of unstimulated UCCs (Fig. 2E) or more distally within the secretory tails of stimulated UCCs (Fig. 2F).

Cytochemistry

Ciliated cells of unstimulated UCCs contained glycogen as indicated by a light PAS staining abolishable by diastase digestion. These cells also contained granular, PAS-positive, and diastase-resistant deposits that measured 0.3–0.5 μm in diameter and were confined to the concave side of the ciliated cell (Fig. 3A). Both intracellular glycogen and granules decreased markedly after exposure of the UCC to serum (Fig. 2D). Numerous granules that also stained with PAS after diastase were demonstrated in third-type cells. These granules measured 0.4–0.6 μm in diameter and were still present in various amounts in stimulated UCCs. Both ciliated and vesicle cells contained variable amounts of lipid droplets (Fig. 3B). These droplets were more frequent in vesicle cells, and their staining reaction was abolished by pretreatment with lipid solvents (Fig. 3C).

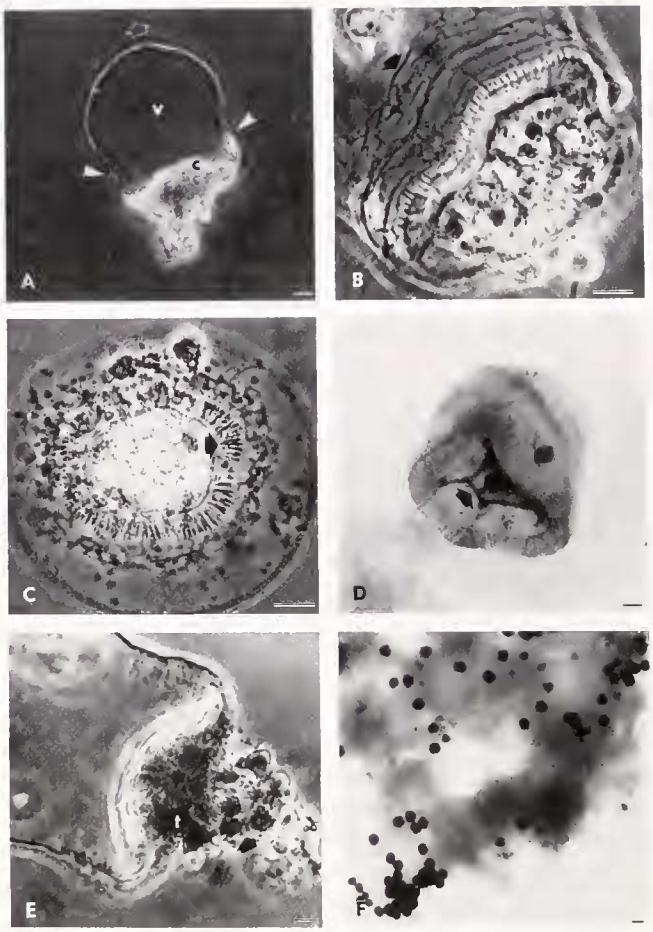

Figure 2. Light microscopy of the urn cell complex (UCC) before (A–C) and after (D–F) exposure to serum. (A) Living UCCs showing the apical vesicle cell (v) which fits into the basal ciliated cell (c), much like an acorn into its cap (arrows). Direction of UCC swimming motion is indicated by empty arrow. (Phase-contrast.) (B) Parallel and refractile rows of filaments (arrow) on the outer aspect of the ciliated cell in a living UCC. (Phase-contrast.) (C) Tail-on view of a living UCC. Note rib-like cytoplasmic insertions of cilia (arrow). (Phase-contrast.) (D) Oblique view of a serum-treated UCC after periodic acid-Schiff (PAS) staining and diastase digestion. Note PAS-positive, putative glycoproteinaceous material (arrow), delimiting a mouth-like opening at the inner periphery of the ciliated cell. A faintly stained secretory tail can also be seen. (E) Lateral view of a UCC fixed 5 min after exposure to serum and stained with methylene blue. The emerging tail (t) stains metachromatically and is pushing distalward a group of third-type cells (arrow). (F) Leading front of a UCC secretory tail 20 min after serum stimulation. Note numerous third-type cells. (PAS.) All scale bars: 10 μm.

The tails of stimulated UCCs stained metachromatically with methylene blue (Fig. 2E) and with PAS after diastase digestion (Fig. 3D). The intensity of the PAS staining varied from mild to marked in different UCCs. In stimulated UCCs, the innermost lining of ciliated cells appeared as a thick and somewhat fuzzy outline that was not abolished by diastase after PAS staining (Fig. 2D). Released tails fluoresced brightly after incubation with FITC-RCA1 and

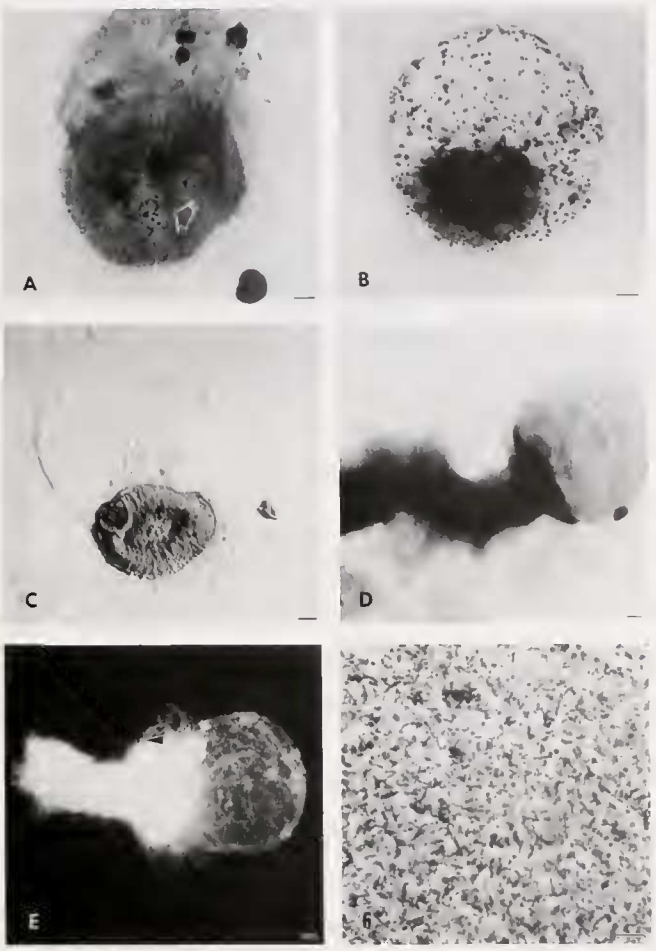

Figure 3. Cytochemistry of the urn cell complex (UCC). (A) PAS-positive, putative glycoproteinaceous material in the ciliated cell component of an unstimulated UCC (arrow). Note that this material is distributed predominantly toward the inner aspect of the UCC. (PAS after diastase digestion.) (B) Unstimulated UCC showing abundant lipid deposits (black) both in the vesicle and ciliated cells. (Sudan black B.). (C) Unstimulated UCC stained with Sudan black B after lipid extraction. Note absence of lipid deposits. (D) UCC 20 min after exposure to serum. The long secretory tail contains PAS-positive material. (PAS after diastase digestion.) (E) Secretory tail 20 min after serum treatment and after incubation with FITC-LTA. Bright fluorescence indicates the presence of fucose-rich moieties. (F) Fibrillary structure of secretory tail. (Methylene blue.) All scale bars: 10 μm.

LTA lectins (Fig. 3E). This fluorescence was specific since it was markedly reduced by D-galactose or L-fucose. Tails stained with methylene blue revealed a fibrillar or fibrillar-granular structure (Fig. 3F).

Ultrastructure

The structural relationship between the vesicle and ciliated cell of the UCC was best appreciated in longitudinal sections (Fig. 4A–B). Both cells were marginally joined by desmosomes (Fig. 4B, inset). The vesicle cell was characterized by a thin cytoplasm containing vacuoles, lipid droplets, and mitochondria (Fig. 5A–B). A single and elongated nucleus was present, usually in the midportion of the cell. As a result of processing for electron microscopy, the vesicle cell frequently appeared collapsed. The bubble-like space enclosed by this cell contained a diffuse electron-dense, fibrillar material (Fig. 5B). No periodicity was observed in individual fibrils. The electron density and fibrillar composition of this material resembled the matrix of the material seen near the mouth-like underside of UCCs (Figs. 4B and 10A–B).

The ciliated cell spanned the entire lower aspect of the UCC. The outer lining of this cell contained numerous prominent microvilli measuring from 0.2–0.4 μm in width and from 1–3 μm in length (Figs. 4A and 6A–B). Straight filaments were seen to run longitudinally inside each microvillus and extend downward into the adjacent ectoplasm (Fig. 6B). Rows of cilia were present between the microvilli. These cilia, 5–10 μm long, with a characteristic 9 + 2 microtubule arrangement (Fig. 6A–B), were anchored onto the underlying cytoplasm by basal bodies and by long (3–5 μm), curving rootlets with a periodicity of 65 nm (Fig. 6C). Away from the ciliary attachment, the cytoplasm contained prominent bundles of 14- to 17-mm-wide microfilaments (Fig. 6D). These appeared to correspond to the filamentous "lines" observed in the neck region of living UCCs (Fig. 2B). In addition to these circular bundles, other smaller bundles of microfilaments and individual microfilaments were seen to traverse the cytoplasm of ciliated cells (Fig. 6C–D). The remainder of the cytoplasm not occupied by cytoskeletal elements contained diffuse glycogen particles, alone (Fig. 7A) or in aggregates (Fig. 7B); mitochondria (Figs. 7C and F); lysosome-like bodies with electron-dense cores (Fig. 6C); and lipid droplets (Figs. 7F–G). Profiles of granular endoplasmic reticulum and Golgi complex were not prominent.

Some of the most prominent structures seen in ciliated cells were irregular vacuoles of various size (Fig. 4A–B). These vacuoles contained no demonstrable products and were surrounded by deposits of electron-dense material that did not appear membrane-bound (Fig. 7C–D and G). A material of similar structure and density was present in the ectoplasmic regions of the ciliated cells that faced the inner neck cavity of the UCC (Fig. 7D–G). This material appeared to cross the cell membrane and merge with fibrillar material that was present in the extracellular space (Fig. 7E–G).

Third-type cells were present below the ciliated cell of the UCC (Figs. 4B and 8C). The former cells were closely juxtaposed to the ciliated cell (Fig. 8A), although no obvious junctional devices were observed between these cells (Fig. 8B). Third-type cells also appeared partly surrounded by small amounts of fibrillar material (Fig. 4B) and cell

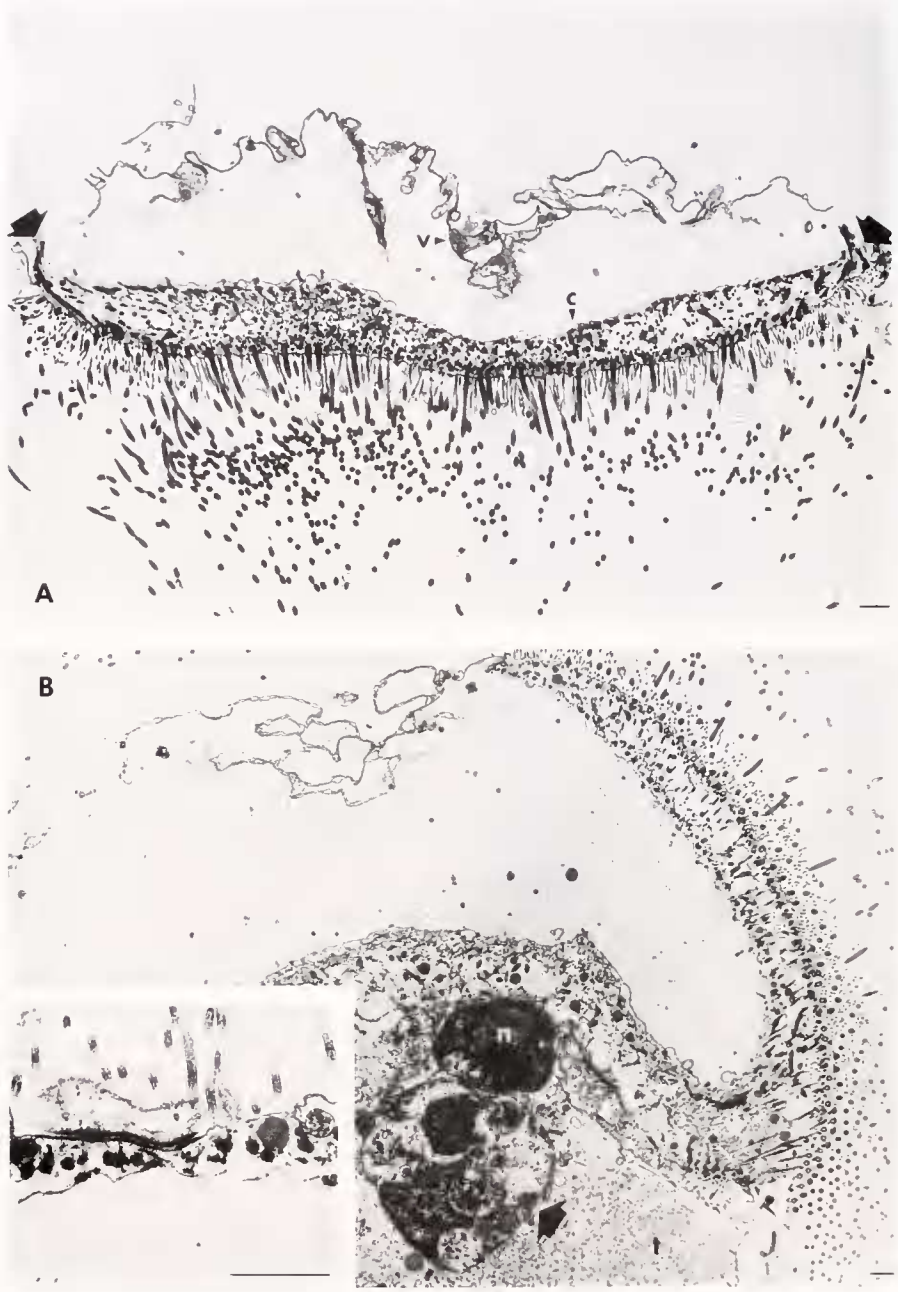

Figure 4. Longitudinal (A) and oblique (B) views of the urn cell complex (UCC). (A) Note partially collapsed vesicle cell (v), ciliated cell (c), and curving bundles of filaments at the junction between these two cells (arrows) before exposure to serum. The ciliated cell contains vacuoles and abundant granular material. (B) Stimulated UCC. The cytoplasm of the ciliated cell contains mostly vacuoles and lipid droplets. Note also ciliated cell nucleus (n), adjacent third-type cells (arrow), and secretory tail (t). Ciliated and vesicle cells are complexed by desmosomes (inset). All scale bars: 1 μm.

debris (Fig. 8C). These cells had an irregular nucleus, and their cytoplasm contained few phagocytic vacuoles and many granules, 0.4–0.6 μm in diameter, with electron-dense cores (Fig. 8C).

To analyze the cytology of stimulated UCCs, these cells were studied after 2, 10, and 20 min of exposure to 1:10 heated serum. The initial formation of a secretory tail was heralded by numerous round elevations of the cell membrane in the underside of the ciliated cell (Fig. 9A). These bleb-like structures had a width of 0.3–0.4 μm and appeared to correspond to underlying ectoplasmic vacuoles (Fig. 9B–C). Ten minutes after stimulation, UCCs exhib-

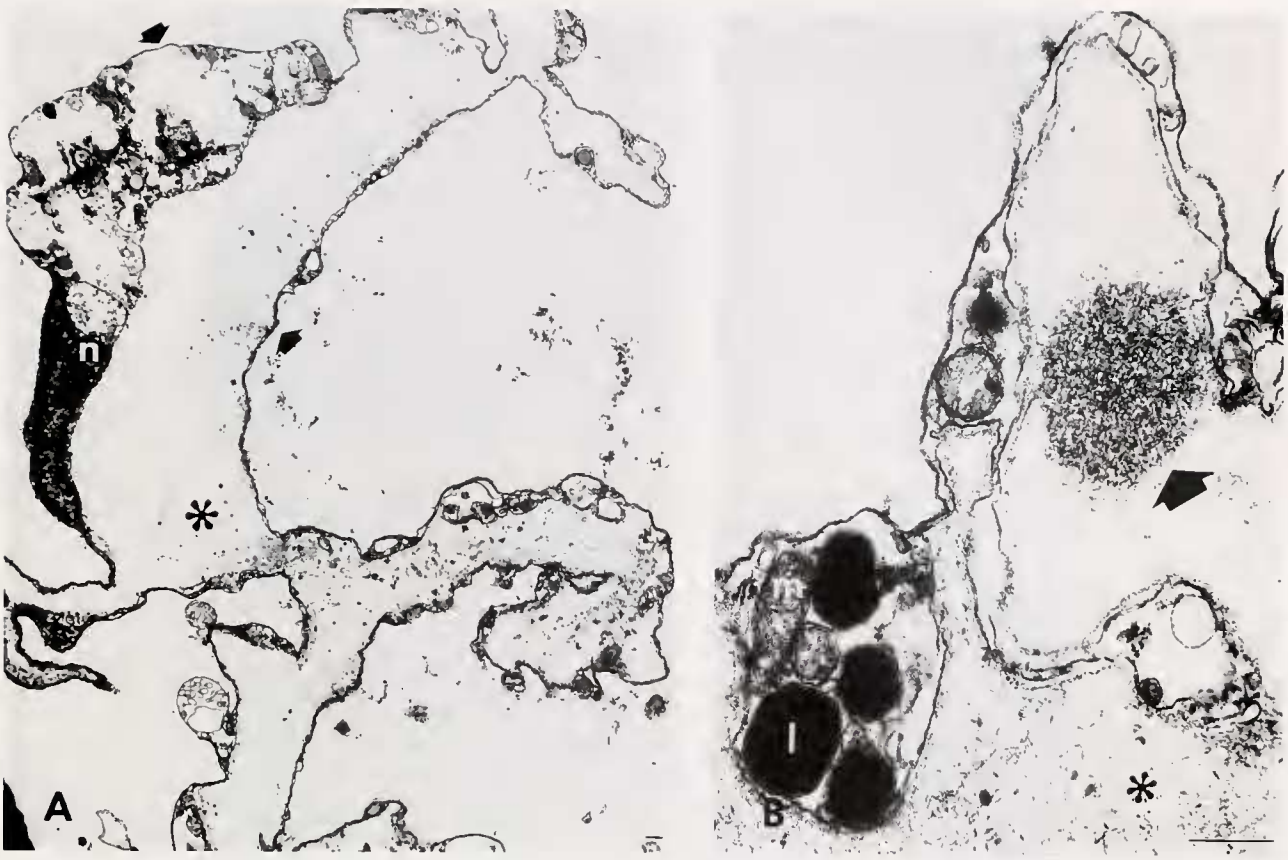

Figure 5. Vesicle cell of the urn cell complex (UCC). (A) Note external surface (arrows) and central space (asterisk). Partial collapse of cell is due to fixation. n, nucleus. (B) Filamentous material in dispersed units (asterisk) or in aggregates (arrow) is present within the UCC central space. Note also lipid droplets (l) and mitochondria (m) within the vesicle cell. Both scale bars: 0.5 μm.

ited abundant extracellular material in this region. The material had a fibrillar-granular structure and was continuous with fibrillar strands originating from ectoplasmic areas of the inner aspect of ciliated cells (Fig. 9D) and from distended bleb-like structures (Fig. 9E). Electron-dense granules were also observed among extracellular fibrils and immediately above the cell membrane (Fig. 9D). Tail-on views of UCCs in transmission and scanning electron micrographs showed that the fibrillar material encircled the inner circumference of the ciliated cell (Fig. 9F–G). This cell contained many empty vacuoles and lipid droplets, little glycogen, and few of the electron-dense aggregates observed in UCCs at rest around intracellular vacuoles and below the cell's plasma membrane. Prominence of intracytoplasmic vacuoles was also obvious in UCCs examined 20 min after exposure to serum (Fig. 10A). When seen in appropriate longitudinal sections, the released tail contained a fibrillar material and occasional third-type cells (Fig. 10B). The electron density and structure of the tail were similar to those of the fibrillar material present within the bubble-like cavity of the UCC (Fig. 10A).

Discussion

The present study analyzed the cytology of free-swimming urn cell complexes of the benthic coelomate *Sipunculus nudus*. The two large cells, ciliated and vesicle, of the UCC are complexed by marginal desmosomes. A smaller and variable population of cells (third-type cells) is frequently, but not invariably, found in close juxtaposition to the ciliated cell. Exposure of a UCC to serum causes a prompt and massive release of a fibrillar-granular material that appears first in the concave side of the ciliated cell and elongates rapidly to form a characteristic tail. During and after release of this tail, increasing cytoplasmic vacuolization occurs in the ciliated cell, together with an apparent transmembranous discharge of fibrillar material and some loss of glycogen. When observed, third-type cells exhibit phagocytic vacuoles and are displaced distalward by the elongating fibrillary tail. Exocytosis cannot be demonstrated in either ciliated or third-type cells.

To this date, the cytology of the UCC of *S. nudus* has been investigated almost exclusively by light microscopy.

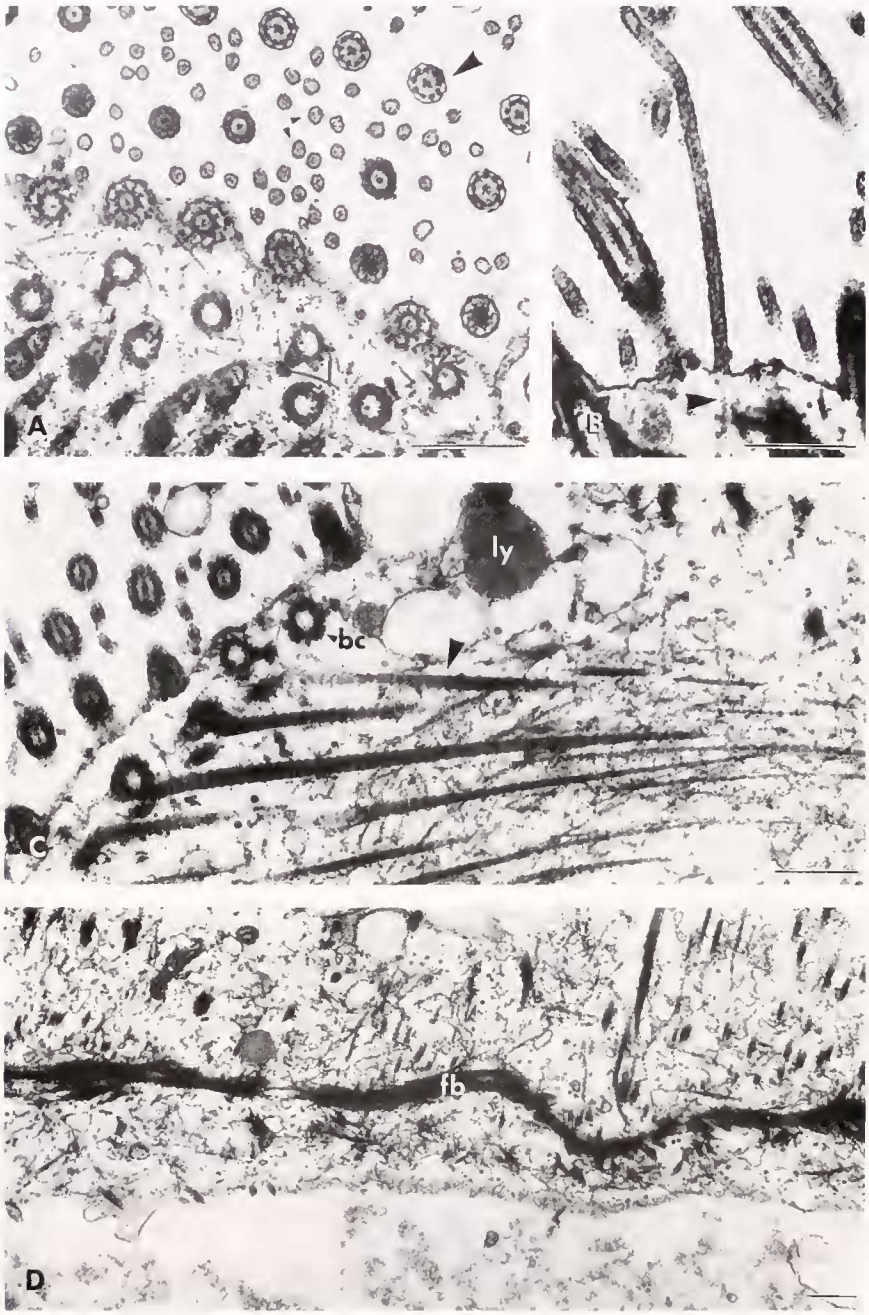

Figure 6. Ciliated cell of the urn cell complex (UCC). (A) Cross-section view of cilia revealing typical spoke-wheel morphology (large arrow) and microvilli (small arrow). (B) Longitudinal view of cilia and microvilli. Note filamentous extension from the core of a microvillus into ectoplasmic area (arrow). (C) Basal corpuscles (bc) and prominent rootlets (arrow) of cilia. Note also individual filaments crossing the cytoplasm in various directions. ly, lysosome-like body. (D) Filamentous bundles (fb) at the outer periphery of the ciliated cell. These prominent bundles run parallel to the cell membrane and correspond to the refractile concentric rows observed in the living UCC (see Fig. 2B). All scale bars: 0.5 μm.

These studies have indicated that not only ciliated and vesicle cells but also a third cell population, so-called R cells, are integral components of the UCC (Reissig *et al.*, 1979; Bang and Bang, 1976, 1980). It has been suggested that R cells may slowly secrete a type of mucus that is selectively sticky for cell debris and foreign particulates (Bang and Bang, 1980). During exposure of a UCC to bacteria or to other foreign substances, R cells become

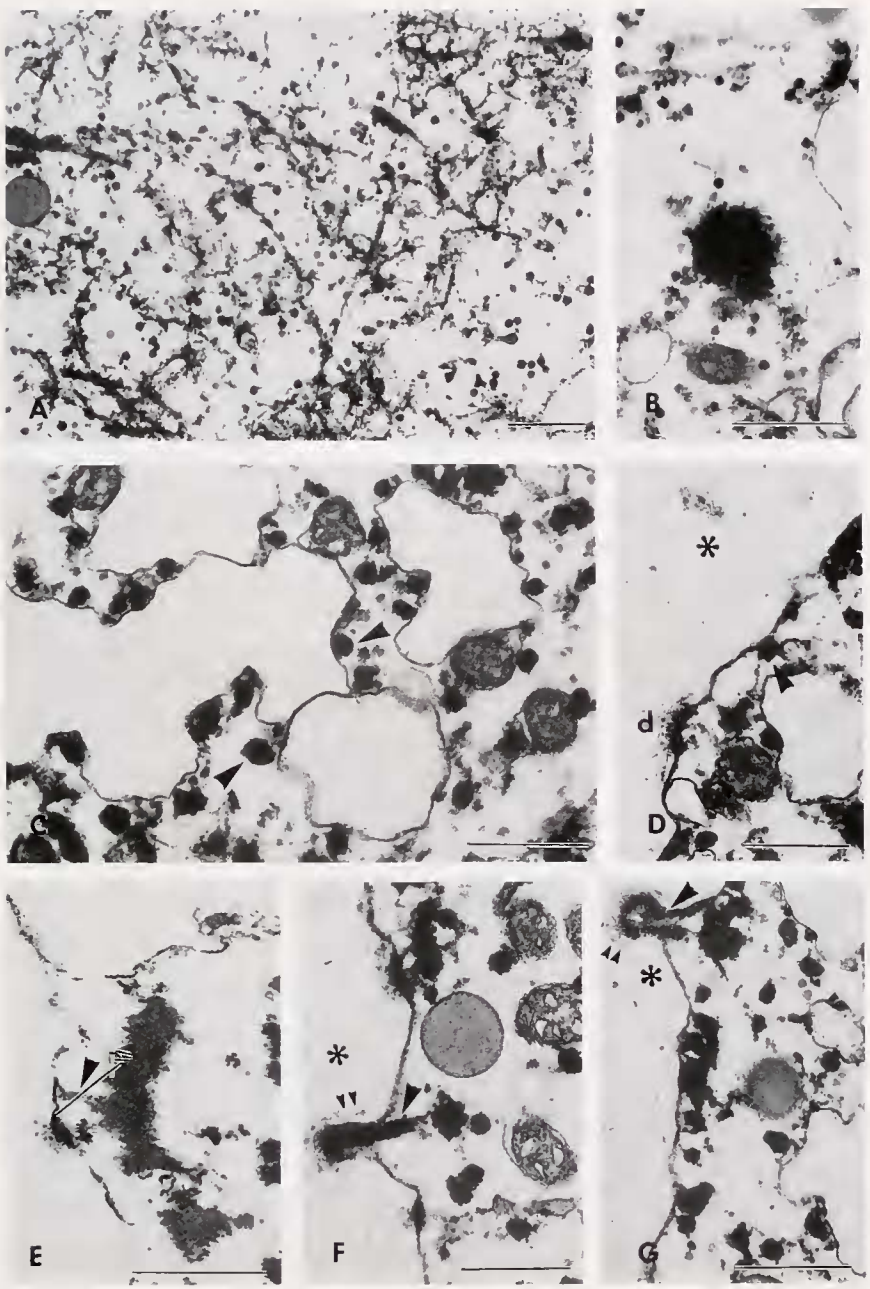

Figure 7. Ciliated cell of the unstimulated urn cell complex UCC. (A–B) Glycogen as individual particles (A) or in aggregate form (B). (C) Electron-dense granular material (arrows) around vacuolar spaces. (D) Similar material (arrow) in close proximity with the ciliated cell membrane facing the UCC central space (asterisk). d, hemidesmosome. (E–G) This material makes frequent contact with the ciliated cell's plasma membrane (arrow) and appears continuous (double arrows) with extracellular fibrillar material of similar electron density (asterisk). All scale bars: 0.5 μm.

detached from the UCC while large streams of mucus are released by the ciliated cell. The loss of R cells is thus accompanied by an apparent progressive increase in the synthesis and release of mucus by the ciliated cell. These observations have suggested that a dual secretory system may exist in the UCC (Bang and Bang, 1980). The first system provided by R cells would be operational at rest. The second system, represented by the ciliated cell, would normally be regulated or held in check by R cells and would become operational during conditions causing hypersecretion and loss of R cells. The present morphological study did not find clear evidence of a dual secretory system

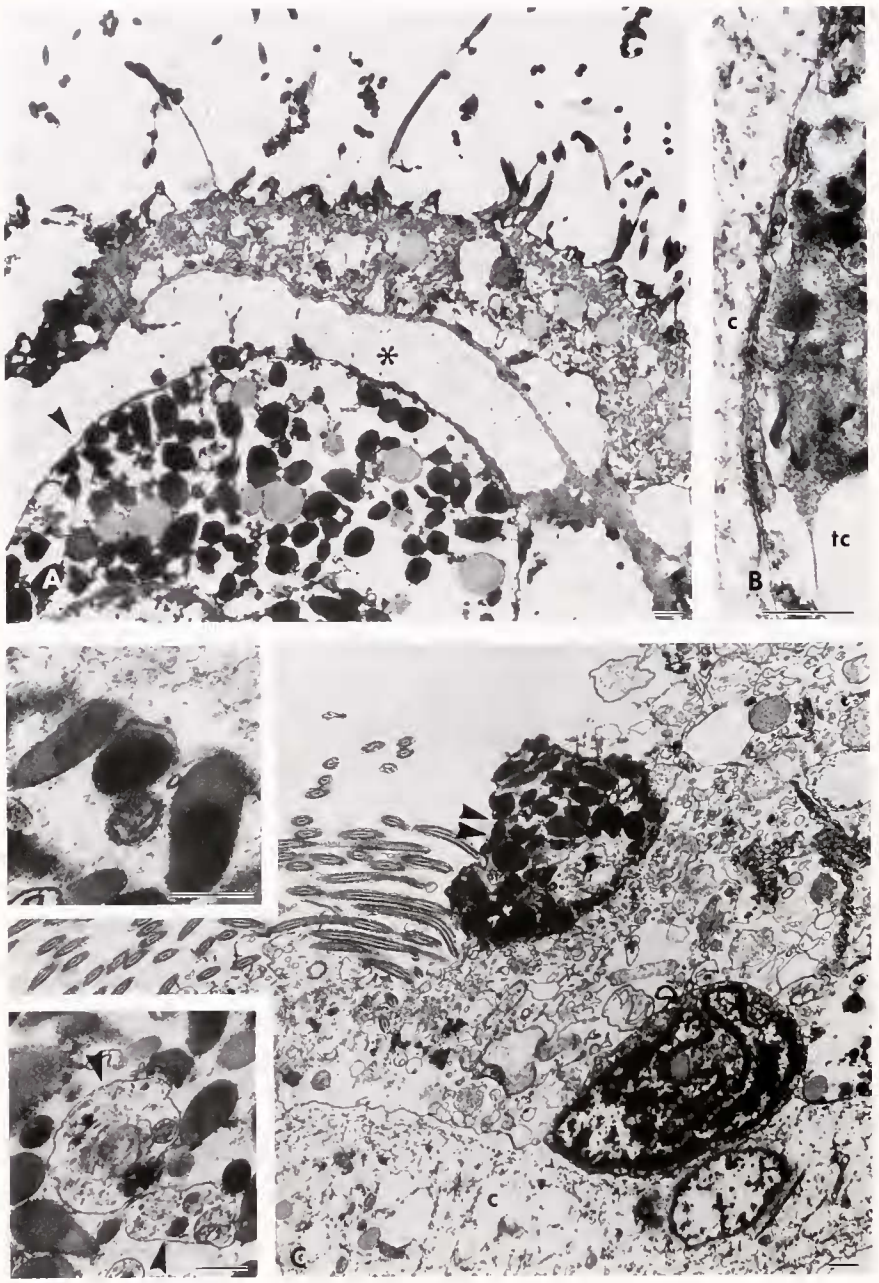

Figure 8. Third-type cell of the urn cell complex (UCC). (A) These cells (arrow) can also be found inside the UCC central space (asterisk). (B) Juxtaposition between ciliated (c) and third-type cell (tc). (C) Two third-type cells 20 min after serum stimulation. The more distal cell (double arrow) contains abundant, lysosome-like granules (upper inset) exhibiting electron-dense cores. Phagocytic vacuoles are also present (lower inset, arrows). c: ciliated cell. All scale bars: 0.5 μm.

in the UCC since release of secretory material from third-type cells could not be documented ultrastructurally at rest or during serum stimulation. Most of the granules of third-type cells had a heterogeneous content and resembled those described in coelomic granulocytes of *Sipunculus nudus* by Valembois and Boiledieu (1980). As in granulocytes, granules of third-type cells often coalesced and became associated with degenerated organelles or engulfed material, indicating phagocytosis. Similar granules, containing enzymes implicated in bacterial killing or in intracellular digestion, have also been described in the coelomic granulocytes of a different sipunculid worm (Dybas, 1981a, b) and in tunicate hemocytes (Sawada *et al.*, 1993).

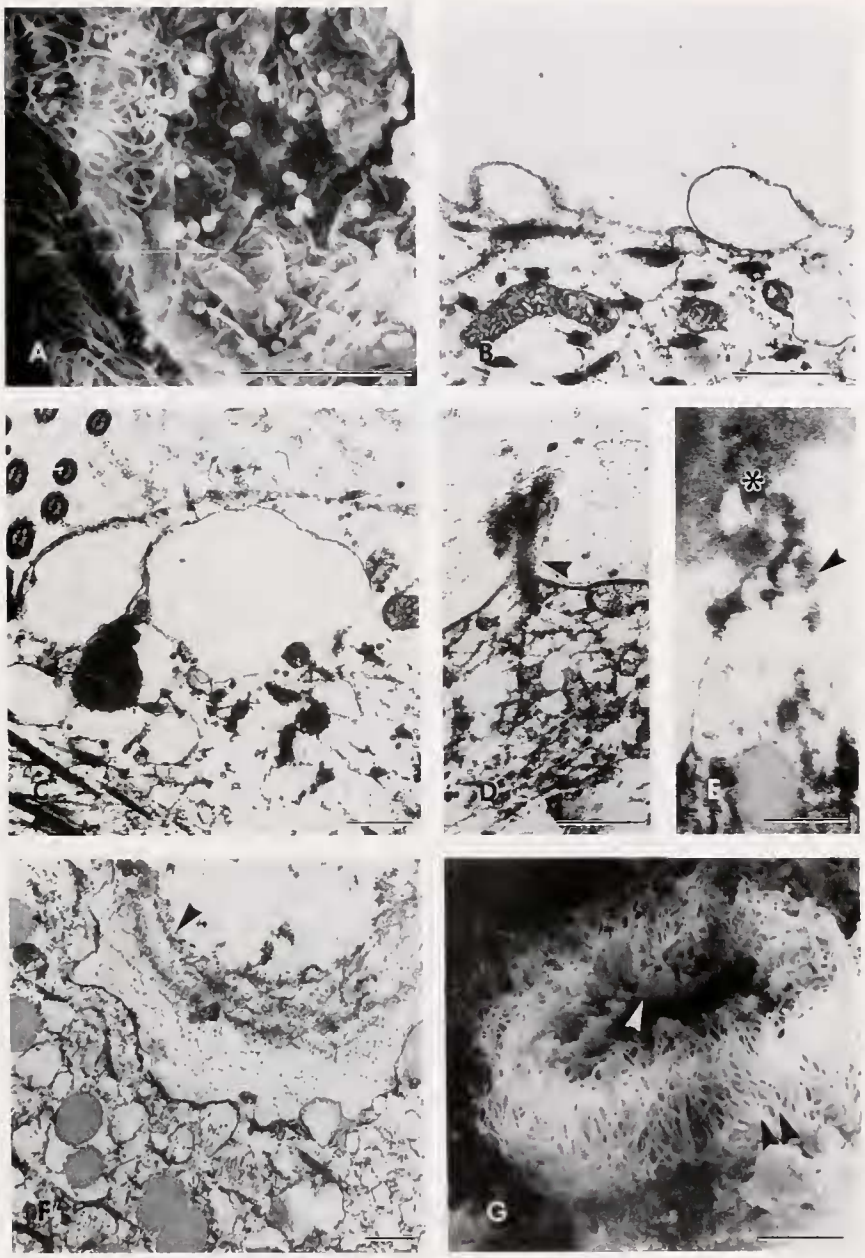

Figure 9. Stimulated urn cell complex (UCC). (A–B) (2 min after exposure to serum). Note bleb-like elevations of cell membrane at the inner side of the ciliated cell. (C) Vacuoles in ciliated cell. (D–E) Tail-on views of UCCs 10 min after exposure to serum. Note electron-dense, fibrillar and granular material seemingly crossing the ciliated cell membrane (D, arrow) or extruding from a distended bleb-like structure (E, arrow) to merge with similar material in a forming tail (E, asterisk). (F–G) Fibrillar material (arrows) surrounding the inner aspect of the mouth-like opening of a UCC 20 min after exposure to serum. Most of the UCC tail has been mechanically removed prior to fixation to better reveal the inner side of the cell. Note also, peripheral and concentric rows of cilia (double arrow). Scale bars: A and G, 10 μm; B–F, 0.5 μm.

Although the present study did not exhaustively characterize either the ultrastructure or the cytochemistry of third-type cells, we suggest that these cells may be macrophages. Clearly, this possibility must be confirmed by functional analysis of living cells and by cytochemical evaluation of third-type cells. We believe that the juxtaposition of third-type cells with the ciliated cell of the UCC does not contradict the phagic nature of third-type cells, since a similar relationship occurs in other mucociliary systems (Jeffery and Reid, 1975; Odor, 1974). The

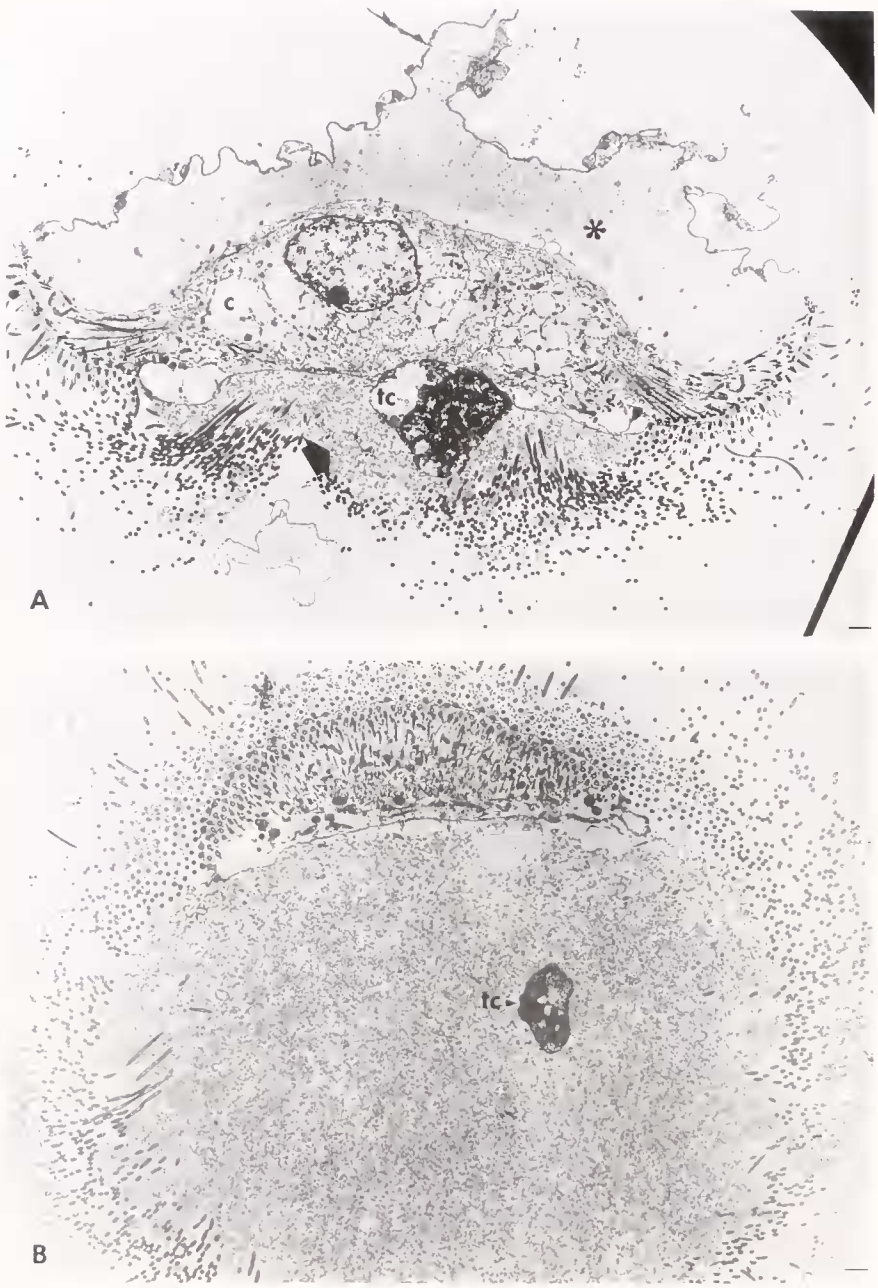

Figure 10. Longitudinal sections of the urn cell complex (UCC) 20 min after exposure to serum. (A) Note moderately electron-dense material within the UCC central space (asterisk) and tail (arrow). c, ciliated cell; tc, third-type cell. (B) Abundant, electron-dense material in released tail of a UCC. tc, third-type cell. Both scale bars: 1 μm.

diversity of cell types in the UCC also suggests some degree of specialization. It is reasonable to assign secretory and locomotory functions to the ciliated component of the UCC. Cilia may also assist the UCC to trap foreign matter and organisms. Third-type cells may phagocytize trapped matter and secrete antibacterial lysins (Bang and Bang, 1962; Dybas, 1981a). These cells may also regulate local secretion (Bang and Bang, 1980). Because the incubation medium we used contained 2% of sipunculid coelomic fluid, the possible contribution of third-type cells and other circulating cellular constituents to tail formation must be investigated further. It is of interest that a lymphoblastoid cell line produces trypsin-sensitive substances that stimulate mucus release in the UCC (Kuleman-Kloene *et al.*, 1982). The role of the vesicle-cell component of the UCC is unclear. This cell encloses a bubble-like space containing

diffuse fibrillar material. In UCCs of *Phascolosoma agassizii,* this space contains connecting fibers that may act as a cementing unit to which the various components of the UCC are attached by hemidesmosomes (Dybas, 1976).

The exact composition and precise mode of origin of the tail released by a UCC after serum stimulation have not yet been determined. Until now, it has been maintained that UCC tails have the physicochemical properties of mucus, including viscoelasticity, structure, and mucoprotein content (Bang and Bang, 1978). However, mucolytic agents that reduce disulfide bonds do not consistently solubilize tails of UCCs (Bang and Bang, 1980). Typical mucous secretory granules have not been detected in the ciliated UCC component of two different sipunculid species: *Sipunculus nudus* (this report) and *Phascolosoma agassizii* (Dybas, 1976). Instead, we have observed in this cell numerous deposits of PAS-positive and diastase-resistant fibrillar material. This material was often seen distending the ciliated cell membrane and migrating across it to merge with similar material present within the UCC cavity. A possibility worth investigating is that this filamentous material is related to fibrin and that released tails are not mucous but fibrinous. Certainly, a fibrinous clot may act as well as mucus in trapping foreign matter, and the coelomic fluid of sipunculids has remarkable clotting properties (Dybas, 1981b). The mechanisms regulating the release of UCC tails also remain to be clarified. The marked depletion of glycogen stores in the ciliated cell suggests a requirement for energy supply. It is not clear if synthesis and secretion of tails are coupled or uncoupled processes, although the sustained production of long tails under certain stimuli supports the former possibility (Bang and Bang, 1972).

The urn cell complex of *Sipunculus nudus* is an interesting model for studying invertebrate and vertebrate mucosal defense mechanisms. Work in our laboratory indicates that rabbit endocervical cells respond in similar fashion *in vivo* and *in vitro* to serum and antigenic challenges, suggesting that the defense systems of invertebrates and vertebrates are phylogenetically convergent (Nicosia, 1986; Nicosia *et al.,* 1982).

Acknowledgments

This investigation was partly supported by USPHS Grant HD-06274 Sub-4 during the senior author's (S.V.N.) stay at the University of Pennsylvania. Live specimens of *Sipunculus nudus* or urn cell complexes were obtained through the courtesy of the late Dr. Frederick B. Bang (School of Hygiene and Public Health, Johns Hopkins University, Baltimore, MD) and Dr. Alberto F. Monroy (Zoological Station of the University of Naples, Italy) to both of whom this article is dedicated. The invaluable assistance of Ms. Young Hae Hyun and Mr. Edward Haller with preparation of the microphotographs and Ms. Neisha P. Sun and Mrs. Lydia Hagenkotter with preparation of the manuscript are also gratefully acknowledged.

Literature Cited

Bang, F. B., and B. G. Bang. 1962. Studies on *Sipunculus* blood: Immunologic properties of coelomic fluid and morphology of "urn cells." *Cah. Biol. Mar.* **3:** 363–374.

Bang, B. G., and F. B. Bang. 1964. Mucus hypersecretion in a normally isolated non-innervated cell. *Cah. Biol. Mar.* **6:** 257–264.

Bang, B. G., and F. B. Bang. 1972. Mucus hypersection induced in isolated mucociliated epithelial cells by a factor in heated serum. *Am. J. Pathol.* **68:** 407–422.

Bang, B. G., and F. B. Bang. 1974. Invertebrate model for study of macromolecular regulators of mucus secretion. *Lancet* ii: 1292–1297.

Bang, B. G., and F. B. Bang. 1975. Cell recognition by mucus secreted by urn cells of *Sipunculus nudus. Nature* **253:** 634–635.

Bang, B. G., and F. B. Bang. 1976. The mucous secretory apparatus of the free urn cell of *Sipunculus nudus. Cah. Biol. Mar.* **17:** 423–432.

Bang, B. G., and F. B. Bang. 1978. Successive histochemical changes in mucus secreted by individual cells. *Fed. Proc.* **37:** 266a.

Bang, B. G., and F. B. Bang. 1979. Mucus-stimulating substances in human body fluid assayed in an invertebrate mucous cell system. *Johns Hopkins Med. J.* **145:** 209–216.

Bang, B. G., and F. B. Bang. 1980. The urn cell complex of *Sipunculus nudus:* a model for study of mucus-stimulating substances. *Biol. Bull.* **159:** 571–581.

Catacuzéne, J. 1928. Réchérches sur les réactions de immunité chéz les invertebres. Réactions de immunité chéz *Sipunculus nudus. Archiv. Roum. Pathol Exp Microbiol.* **1:** 7–80.

Chantler, E., and E. Debruyne. 1977. Factors regulating the changes in cervical mucus in different hormonal states. Pp. 131–141 in *Mucus in Health and Disease,* M. Elstein and D. V. Parke, eds. Plenum Press, New York.

Dybas, L. 1976. A light and electron microscopic study of ciliated urn of *Phascolosoma agassizii* (sipunculid). *Cell Tissue Res.* **169:** 67–75.

Dybas, L. 1981a. Sipunculids and echiuroids. Pp. 161–188 in *Invertebrate Blood Cells.* Vol 1. N. A. Ratchliffe and A. F. Rowley, eds. Academic Press, New York.

Dybas, L. 1981b. Cellular defense reactions of *Phascolosoma agassizii,* a sipunculan worm: phagocytosis by granulocytes. *Biol. Bull.* **161:** 104–114.

Hall, R. L., A. C. Peatfield, and P. S. Richardson. 1978. The effect of serum on mucus secretion in the trachea of the cat. *J. Physiol. (Lond.)* **282:** 477–478.

Hyman, L. H. 1959. *The Invertebrates: Smaller Coelomate Groups.* Vol. 5, pp. 611–695, McGraw-Hill, New York.

Jeffery, P. K., and L. Reid. 1975. New observations of rat airway epithelium: a quantitative and electron microscopic study. *J. Anat.* **120:** 295–320.

Kuleman-Kloene, H., S. S. Krag, and F. B. Bang. 1982. Mucus secretion stimulating activity in human lymphoblastoid cells. *Science* **217:** 736–737.

Kurlandsky, L. E., R. W. Berninger, and R. C. Talamo. 1980. Mucus-stimulating activity in the sera of patients with cystic fibrosis: demonstration and preliminary fractionation. *Pediatr. Res.* **14:** 1267–1268.

Lillie, R. D. 1965. *Histologic Technic and Practical Histochemistry,* p. 38. McGraw-Hill, New York.

Luna, L. G. 1968. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology,* 3rd ed. McGraw-Hill, New York.

Luft, J. M. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9:** 409–414.

Mastroianni, L., Jr., S. V. Nicosia, and E. Streibel. 1979. Studies on urn cell complexes of *Sipunculus nudus* (Linnaeus): influence of physiologic and pathologic mammalian sera on mucus secretion. *Biol. Bull.* **157:** 381a.

Nicosia, S. V. 1979. Lectin-induced mucus release in the urn cell complex of the marine invertebrate *Sipunculus nudus* (Linnaeus). *Science* **206:** 698–700.

Nicosia, S. V. 1986. Physiology of cervical mucus production. *Sem. Reprod. Endocrinol.* **4:** 313–321.

Nicosia, S. V., and J. Sowinski. 1979. Ultrastructure of urn cell complexes of *Sipunculus nudus* (Linnaeus) before and after serum-induced mucus release. *Biol. Bull.* **157:** 385a.

Nicosia, S. V., J. H. Johnson, E. J. Streibel, and L. Mogul. 1982. Serum-induced hypersecretion in isolated and native rabbit endocervical mucous cells. *EMSA* **40:** 244–245.

Odor, L. 1974. The question of "basal" cells in oviductal and endocervical epithelium. *Fertil. Steril.* **25:** 1047.

Reissig, M., B. G. Bang, and F. B. Bang. 1979. Mucus secretion in the urn cell complex of *Sipunculus nudus*. *J. Cell Biol.* **83:** 433a.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17:** 208–212.

Richardson, P. S., and R. J. Phipps. 1978. The anatomy, physiology, pharmacology and pathology of tracheobronchial mucus secretion and the use of expectorant drugs in human disease. *Pharmacol. Ther.* **3:** 441–479.

Sawada, T., J. Zhang, and E. L. Cooper. 1993. Classification and characterization of hemocytes in *Styela clara*. *Biol. Bull.* **184:** 87–96.

Valembois, P., and D. Boiledieu. 1980. Fine structure and functions of haemerythrocytes and leucocytes of *Sipunculus nudus*. *J. Morphol.* **163:** 69–77.